NCBI Primer-BLAST

An online tool for designing target-specific PCR primer pairs (with internal probes)

https://www.ncbi.nlm.nih.gov/tools/primer-blast/

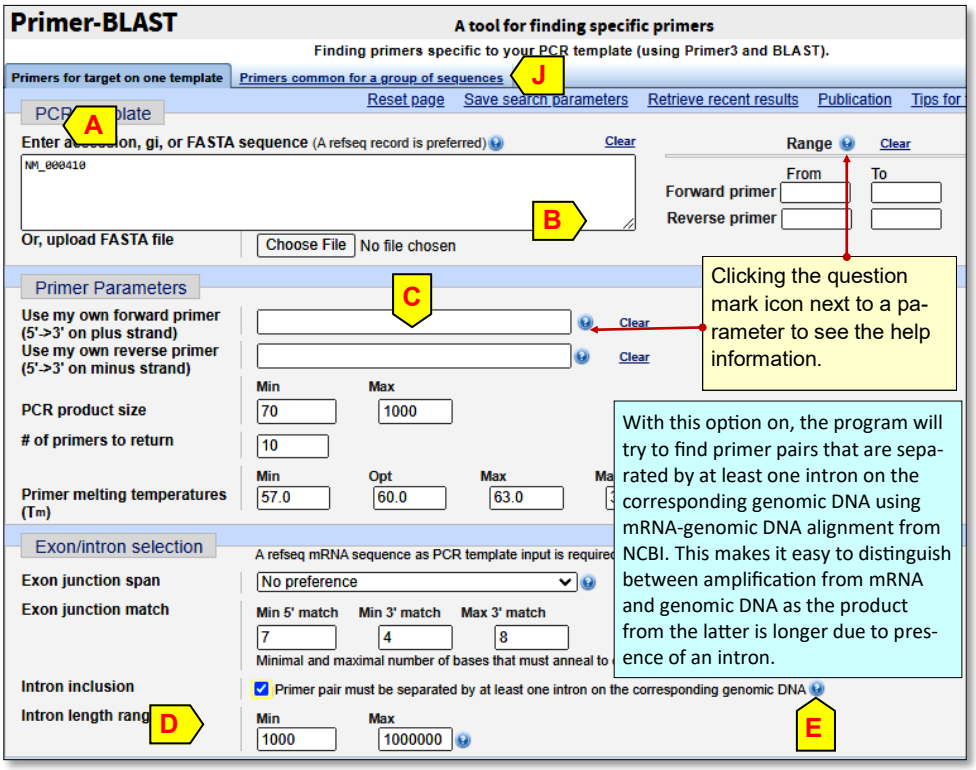
National Center for Biotechnology Information • National Library of Medicine • National Institutes of Health • Department of Health and Human Services

Scope and Access

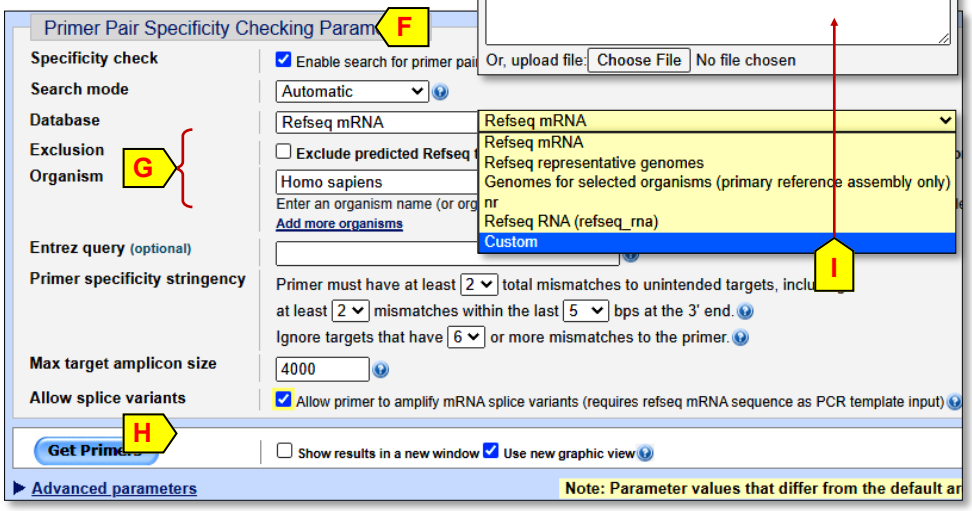
Primer-BLAST [1] is a PCR primer design and specificity checking tool from NCBI. It first scans the database to identify unique regions in the supplied template, picks primers from these regions using the Primer3 algorithm [2], and screens the returned primer sets using BLAST [3] to evaluate their specificity to the input template. It presents candidate primers along with their alignment to identified target sequences in the database. Primer-BLAST is a web only application accessible through the "Specialized BLAST" section of the BLAST homepage (blast.ncbi.nlm.nih.gov/) or directly through the Primer-BLAST search form at www.ncbi.nlm.nih.gov/tools/primer-blast/.

Accepted Inputs

The Primer-BLAST search page (right & below) contains multiple sections. The "PCR Template" (**A**) takes your input template, for which you can limit where the forward and reverse primers has to be picked up from (**B**). With a valid template, Primer-BLAST will find a set of primer pairs optimal for PCR amplification. The "Primer Parameter" accepts different combinations of primer input (**C**), such as a primer pair with its template, a template with a single primer, and a pair of primers alone. With specificity check option selected (default), Primer-BLAST will screen primer pairs for their specificities and report any potential off-target annealing. With a single primer input, Primer-BLAST finds candidate primers working with the input template and primer. For primer pairs only input, Primer-BLAST finds the amplification target in selected organism and return the primer template alignments. With a RefSeq mRNA accession as an input template, Primer-BLAST can take exon junctions into consideration when finding optimal primer pairs, through the intron inclusion option (**D**) in the "Exon/intron selection" section. More information on the parameter is available in the popup (**E**) activated through the question mark click.



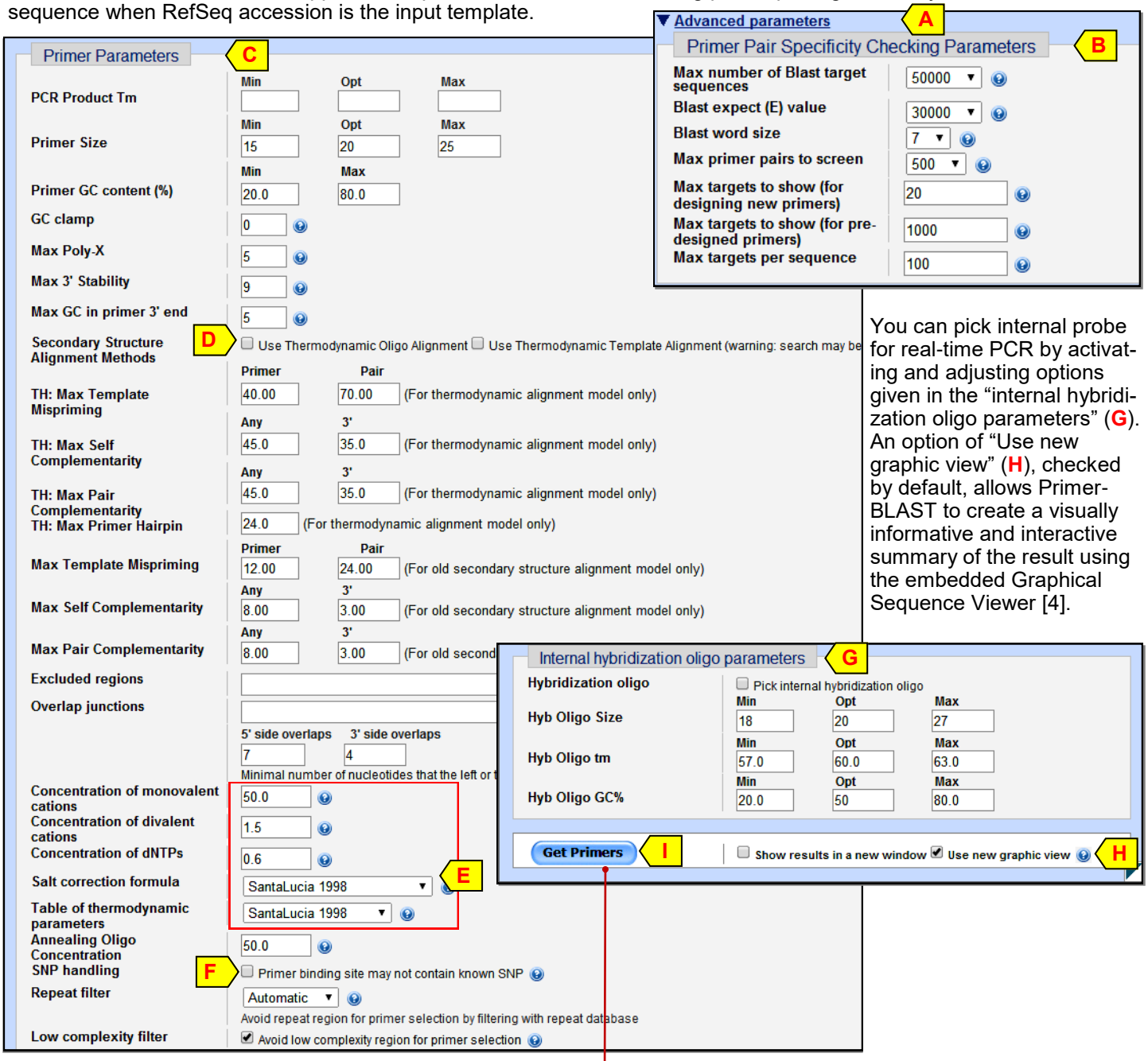
The "Primer Specificity Checking Parameters" section (**F**) provides databases and organism limit selection (**G**). To increase the chance of finding specific primers, check the splice variant option (**H**). Change the database to "Custom" (**I**) option to upload a custom set of sequences (accessions or FASTA) for use as the specificity checking database.



The newly introduced "Primers common for a group sequences" tab (**J**) has the same setup, but the template input box takes a group of similar sequences as input and designs PCR primers that amplify all the input templates.

Advanced Parameters for Primer-BLAST

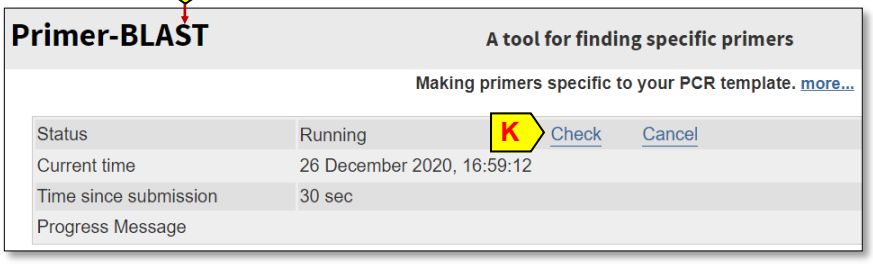
Clicking the “Advanced Parameters” link (**A**) toggles on infrequently adjusted parameters. Parameters in the “Primer Pair Specificity Checking Parameters” (**B**) specify the exhaustiveness of specificity checking and how many results to display. The “Primer Parameters” (**C**) specify the Tm of the product, and specific properties of the returned primers pairs. In favor of search speed, Primer-BLAST does not use thermodynamic alignment features (**D**) by default. Settings in buffer condition can greatly affect the primer Tm calculation and you can adjust them here (**E**). Check the checkbox (**F**) to instruct Primer-BLAST to take SNPs mapped to template into consideration during primer picking. This only works for human sequence when RefSeq accession is the input template.



You can pick internal probe for real-time PCR by activating and adjusting options given in the “internal hybridization oligo parameters” (**G**). An option of “Use new graphic view” (**H**), checked by default, allows Primer-BLAST to create a visually informative and interactive summary of the result using the embedded Graphical Sequence Viewer [4].

Submitting a Search

Click the “Get Primers” button (**I**) to submit the search. The browser tracks the progress of the submitted job via an intermediate polling page (**J**) and displays the result when it becomes available. You can manually check it by using the “Check” link (**K**).

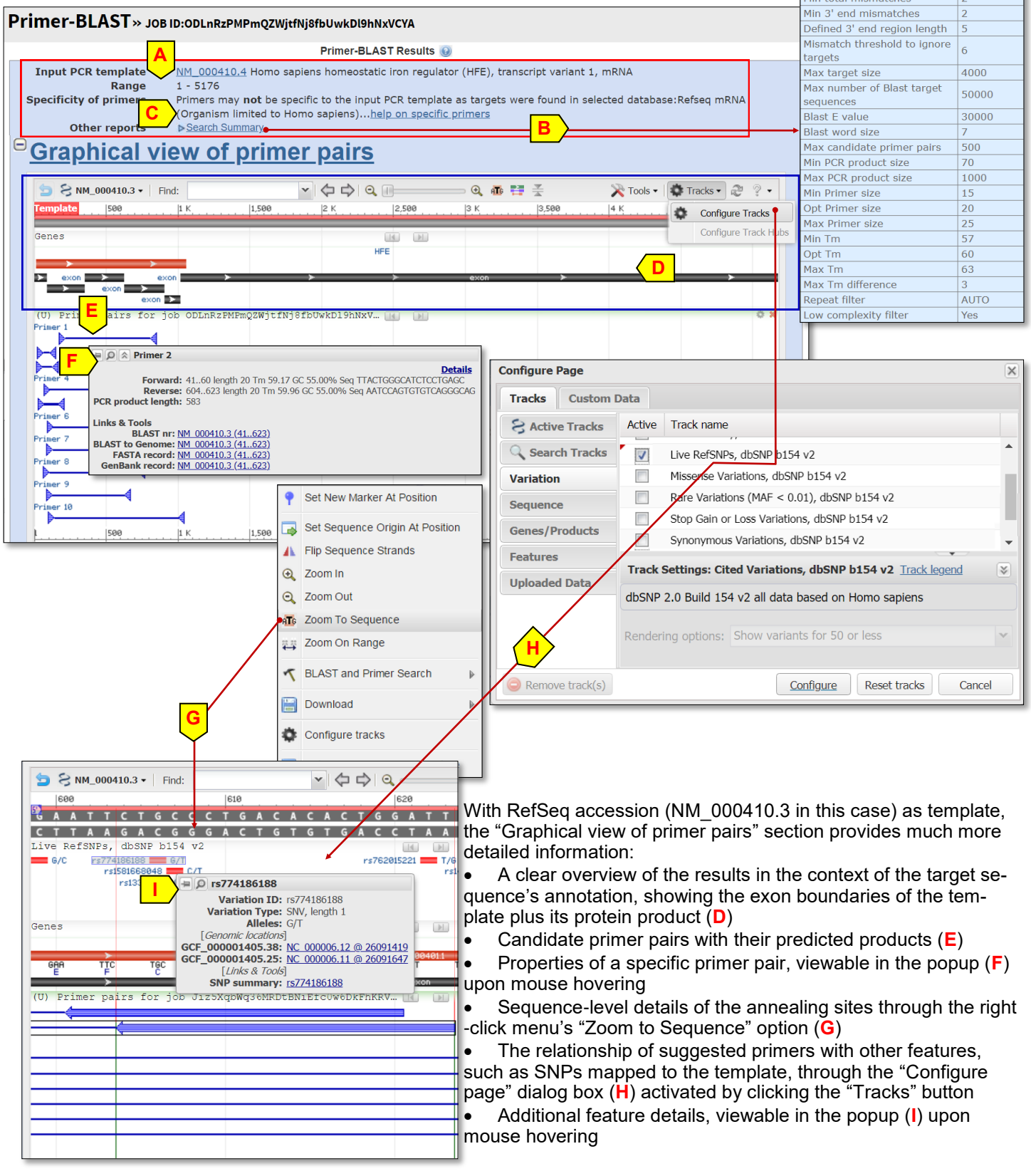


Primer-BLAST Results: the Graphical Summary

The Primer-BLAST displays results by breaking them into the search summary, the “Graphical view of primer pairs”, and a “Detail primer reports” sections.

The summary section reiterates the template (**A**) and provides a “Search Summary” link with detailed statistics of the search (**B**). The informational message (**C**) provides additional details on the primers returned, with a link to online document with tips.

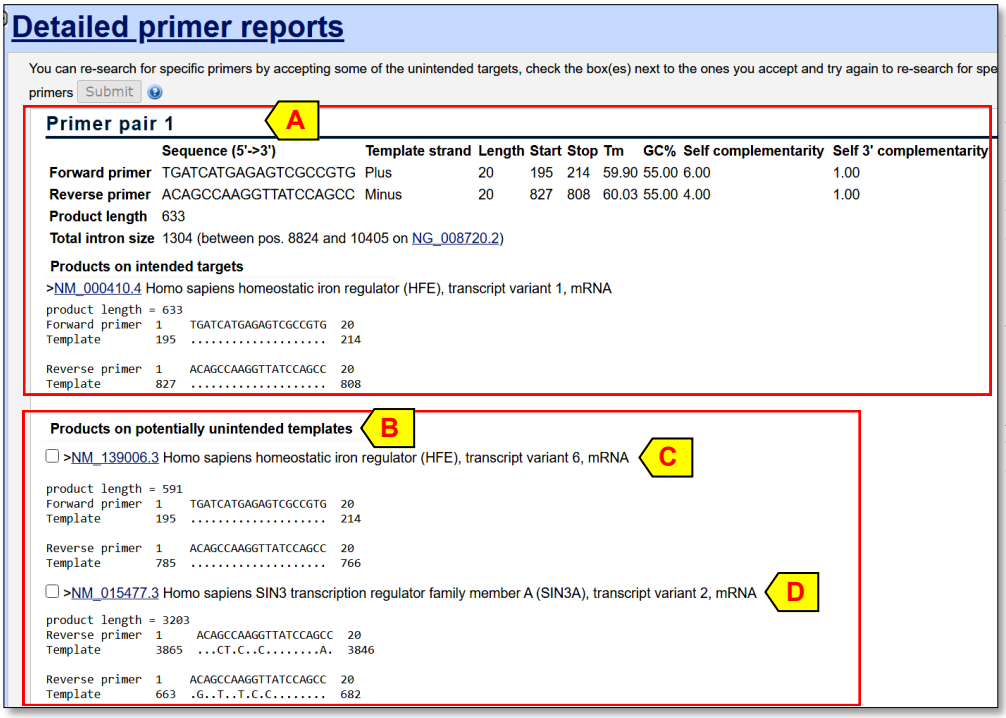
Search parameters and other details	
Number of Blast hits analyzed	338307
Entrez query	
Min total mismatches	2
Min 3' end mismatches	2
Defined 3' end region length	5
Mismatch threshold to ignore targets	6
Max target size	4000
Max number of Blast target sequences	50000
Blast E value	30000
Blast word size	7
Max candidate primer pairs	500
Min PCR product size	70
Max PCR product size	1000
Min Primer size	15
Opt Primer size	20
Max Primer size	25
Min Tm	57
Opt Tm	60
Max Tm	63
Max Tm difference	3
Repeat filter	AUTO
Low complexity filter	Yes



With RefSeq accession (NM_000410.3 in this case) as template, the “Graphical view of primer pairs” section provides much more detailed information:

- A clear overview of the results in the context of the target sequence’s annotation, showing the exon boundaries of the template plus its protein product (**D**)
- Candidate primer pairs with their predicted products (**E**)
- Properties of a specific primer pair, viewable in the popup (**F**) upon mouse hovering
- Sequence-level details of the annealing sites through the right-click menu’s “Zoom to Sequence” option (**G**)
- The relationship of suggested primers with other features, such as SNPs mapped to the template, through the “Configure page” dialog box (**H**) activated by clicking the “Tracks” button
- Additional feature details, viewable in the popup (**I**) upon mouse hovering

Primer-BLAST Results: Primer Pairs and Their Alignment to Targets

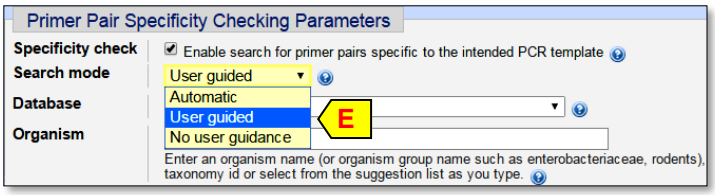


The "Detailed Primer Reports" section (left) contains the details for returned primer pairs. Each primer pair is in its own subsection (**A**), which lists basic primer properties along with alignments to their intended target. Alignments to potentially unintended targets (**B**) are at the end of the subsection.

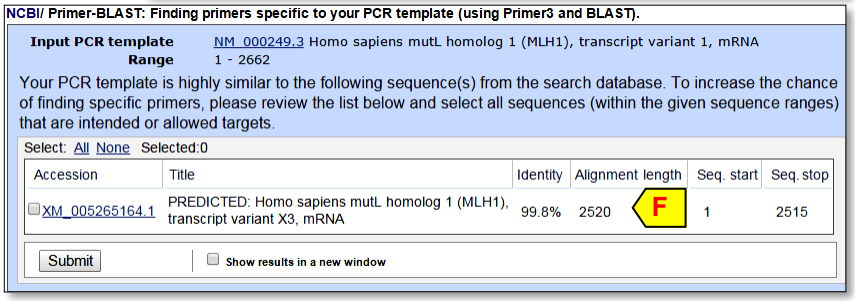
In this example, the first primer pair for human HFE gene transcript variant 1 (NM_000410) also amplify other variants, such as variant 6 (**C**). Alignments (with mismatches) to a truly unintended target SIN3 gene transcripts are also shown (**D**).

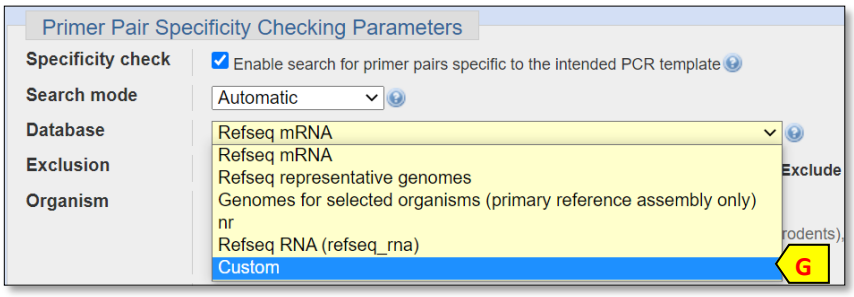
More on "User guided" Mode and "Custom" Database

The search form's "Primer Pair Specificity Checking Parameters" section provides the "User guided" (**E**) mode to allow Primer-BLAST to distinguish between the intended template and other targets that are highly similar to it (such as other transcript variants from the same gene) upon the job submission (**F**).



Selecting custom database (**G**) allows you to provide custom dataset for specificity checking. System constraints limit the size of sequence files to 300 MB. For sequences already deposited in the NCBI Nucleotide database, you can use their accessions to specify a larger custom dataset.





References

1. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. (2012) Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics. 13:134.
2. Rozen, S and Skaletsky, HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) *Bioinformatics Methods and Protocols: Methods in Molecular Biology.* Humana Press, Totowa, NJ, pp 365-386.
3. Altschul, SF, Madden, TL, Schäffer, AA, Zhang, J, Zhang, Z, Miller, W and Lipman, DJ (1997) "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs." Nucleic Acids Res. 25:3389-3402.
4. The Graphical Sequence Viewer Factsheet: https://ftp.ncbi.nih.gov/pub/factsheets/Factsheet_Graphical_SV.pdf.

Technical Assistance

Please send you feedback, questions and bug reports to blast-help@ncbi.nlm.nih.gov